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Effects of environmentally relevant sub-chronic atrazine concentrations on African clawed frog (*Xenopus laevis*) survival, growth and male gonad development

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ABSTRACT

Sub-chronic toxicity of environmentally relevant atrazine concentrations on exposed tadpoles and adult male African clawed frogs (*Xenopus laevis)* was evaluated in a quality controlled laboratory for 90 days. The aim of this study was to determine the effects of atrazine on the survival, growth and gonad development of African clawed frogs. After exposure of tadpoles to atrazine concentrations of 0 (control), 0.01, 200 and 500 $\mu g\,L^{-1}$ in water, mortality rates of 0, 0, 3.3 and 70% respectively were recorded for the 90 day exposure period. Morphometry showed significantly reduced tadpole mass in the 500 $\mu g\,L^{-1}$ atrazine exposed tadpoles ($p < 0.05$). Light microscopy on testes of adult frogs exposed to the same atrazine concentrations using hematoxylin and eosin (H&E) and Van Gieson staining techniques revealed gonadal atrophy, disruption of germ cell lines, seminiferous tubule structure damage and formation of extensive connective tissue around seminiferous tubules of frogs exposed to 200 $\mu g\,L^{-1}$ and 500 $\mu g\,L^{-1}$ atrazine concentrations. Ultrastructural analysis of the cellular organelles using transmission electron microscopy (TEM) revealed significant amounts of damaged mitochondria in testosterone producing Leydig cells as well as Sertoli cells. Biochemical analysis revealed reduced serum testosterone levels in adult frogs at all exposure levels as well as presence of six atrazine metabolites in frog serum and liver. The results indicate that atrazine concentrations greater than the calculated LC50 of 343.7 $\mu g\,L^{-1}$ cause significant mortality in tadpoles, while concentrations $\geq 200\,\mu g\,L^{-1}$ adversely affect reproductive health of adult frogs and development of tadpoles sub-chronically exposed to atrazine.

1. Introduction

Atrazine (CAS# 1912-24-9) is one of the most ubiquitous and extensively used herbicides in the world for the control of broad leaf weeds (Khalil et al., 2017; Schmidt et al., 2017; Zheng et al., 2017). It has unrestricted use in most parts of the world, however its use is banned in the European Union since 2004 (Jablonowski et al., 2011; Yang et al., 2017). The maximum allowable atrazine concentrations in drinking water range from 0.1 to 3 $\mu g\,L^{-1}$ in most regions, particularly in Europe, Asia and America (Singh et al., 2018). A recent monitoring study of atrazine across the Ceará state in Brazil revealed mean atrazine concentrations of 7–15 $\mu g\,L^{-1}$ in reservoir water (Sousa et al., 2016). Atrazine environmental concentrations are sometimes found in much higher concentrations, even over 500 $\mu g\,L^{-1}$ (Freeman and Rayburn, 2005; Giddings et al., 2005; Rohr and McCoy, 2010; Storrs and Kiesecker, 2004; Tavera-Mendoza et al., 2002). Aquatic organisms are likely to be exposed sub-chronically to high atrazine concentrations for periods of up to 3 months in ponds and pools adjacent to fields at the height of the agricultural season as well as chronically to low atrazine concentrations all year round in rivers and lakes downstream (Storrs and Kiesecker, 2004; Wood et al., 2017). Sub-chronic exposures of animals occur over a portion of their life or life cycle (Zhang et al., 2016). Rainfall simulation studies have shown that a high percentage of the atrazine applied on land is lost to ground and surface water sources

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through surface run-off during the first rain after application, with up to 75% loss occurring over 70 days in loam soils and much less in clay soils where it is prone to transformation into a variety of metabolites (Koskinen and Clay, 1997; Ng and Clegg, 1997; Wallace et al., 2017; Wang et al., 2018).

The widespread presence of atrazine and its metabolites in the environment is reported to be the cause of declining amphibian populations worldwide (Forson and Storfer, 2006; Hayes et al., 2010; Moreira et al., 2017; Shipitalo and Owens, 2003; Siddiqua et al., 2010). Results of atrazine exposure studies often produce valid but contrasting conclusions (Brodeur et al., 2009; Du Preez et al., 2008; Gammon et al., 2005; Kloas et al., 2009; Oka et al., 2008; Rohr and McCoy, 2010; Solomon et al., 2008). It has been proposed that the conflicting results may be due to a combined agonist-antagonist atrazine effect e.g atrazine is proven to both accelerate and delay metamorphosis (Rohr and McCoy, 2010). A variety of frog species exposed to high atrazine concentrations had lower occurrence of gonadal dysgenesis, testicular oocyte development and higher survival rates than frogs exposed to much lower atrazine concentrations (Hayes et al., 2003; Jooste et al., 2005; Storrs and Kiesecker, 2004). This phenomenon described by Storrs and Kiesecker, (2004) as a non-monotonic response has been observed in a wide range of animals. The link between atrazine exposure and development of testicular oocytes in different frog species is one that also remains uncertain (McDaniel et al., 2008; Rohr and McCoy, 2010). It has however been proven that tadpoles exposed to atrazine prior to sexual differentiation, develop aplasia and gonadal dysgenesis (Hayes et al., 2002; Tavera-Mendoza et al., 2002).

Studies on different frog species exposed to atrazine concentrations up to 20 $\mu g\,L^{-1}$ (exposure periods from 2 to 75 days) have indicated that there are no significant effects on embryo hatchability and tadpole growth i.e length and mass endpoints (Allran and Karasov, 2001; Diana et al., 2000; Morgan, 1996) and exposures up to 100 $\mu g\,L^{-1}$ for 75 days do not significantly affect sexual differentiation and survival (Kloas et al., 2009). Studies by Brodeur et al. (2009) and Rutkoski et al. (2018) have indicated that pre- and prometamorphosis tadpoles are more sensitive to atrazine than embryos. Design flaws and contamination of controls in some studies have led to questionable results and pronouncement of controversial conclusions (Hayes, 2004; Rohr and McCoy, 2010). Conflicts of interest have also often been singled out as a contributor to questionable results as well as negative results (Hayes, 2004). Trematode and other infections observed in frogs in the environment have been attributed to the presence of atrazine and its metabolites in the aquatic environment (Rohr et al., 2008).

The scope of this work is limited to specific biological and toxicological effects of environmentally relevant atrazine concentrations including a 0.01 $\mu g\,L^{-1}$ concentration selected to represent trace concentrations, a documented no observed effect concentration (NOEC) of 200 $\mu g\,L^{-1}$ for *X. laevis* tadpoles (Langerveld et al., 2009; Rohr and McCoy, 2010) and a 500 $\mu g\,L^{-1}$ concentration which is rarely studied. *X. laevis* was selected for this study as it is a good indicator of environmental pollution due to the frog's semi-aquatic life cycle and properties such as semi-permeable skin. The effect of atrazine on mortality, growth and development of frogs and tadpoles was studied in light of reports that atrazine is responsible for declining global frog populations. The effects of atrazine on qualitative and quantitative subcellular testes morphology and serum sex steroid hormone levels of *X. laevis* were assessed to determine the effects of atrazine on gonad development and feminization of male frogs. It is envisaged that this study will provide an unprejudiced assessment of the effects of atrazine on *X. laevis* that would in combination with other similar studies, allow decision makers to gain an informed perspective on risk assessment of environmental atrazine contamination. A 90 day (sub-chronic) exposure was selected as frogs in the wild are usually exposed during short periods between applications and in most cases within the corn growing season spanning three months, though longer exposures are encountered in some lakes, ponds and pools (Solomon et al., 2008).

2. Materials and methods

2.1. Chemicals

30 $mg\,L^{-1}$ (1.39×10^{-4} M) atrazine stock solutions were made up by dissolving 15 mg atrazine (certified reference standard, purity 99.5%) in 500 mL Milli-Q water. Stock solutions for 17-β estradiol, 17-α estradiol, testosterone, atrazine desisopropyl-2-hydroxyl (AD-2OH), desisopropylatrazine (DIA), atrazine-2-hydroxy (A-2OH) and deethylatrazine (DEA) as well as internal standards D7 deethylatrazine, D4 17-β estradiol and D4 estrone were made up to 1 $mg\,mL^{-1}$ in methanol. Atrazine desethyl-desisopropyl (ADD) and hydroxyatrazine (HA) which have very low solubility in methanol were made up to lower concentrations of 100 $mg\,L^{-1}$ in Milli-Q water: methanol (50:50, v/v). Atrazine stock solution for gas chromatography analysis was made up to 100 $mg\,L^{-1}$ in toluene. All analytical standards had a purity ≥96% and accurate concentrations were made up by gravimetric compensation for standards with < 100% purity.

2.2. Experimental design and treatment

For this study, two separate atrazine exposures were carried out with adult male frogs (between May and August 2015 and March and June 2016) and two concurrent exposures were carried out with tadpoles between May and August 2015. Sixty laboratory bred adult (263 day old) male African clawed frogs (*Xenopus laevis*) were procured from the African Xenopus Facility (Knysner, South Africa) for each duplicate exposure. The frogs were flown to Johannesburg and on arrival, they were acclimatized for 7 days before being randomly distributed into 4 groups of 15 and exposed to 0 (control), 0.01 $\mu g\,L^{-1}$ (4.64×10^{-11} M), 200 $\mu g\,L^{-1}$ (9.27×10^{-7} M) and 500 $\mu g\,L^{-1}$ (2.32×10^{-6} M) atrazine solutions (prepared in water) in 200 L stainless steel tanks. Adult frogs were fed *ad libitum* with nutritious commercial fish pellets (Koi food) and beef liver pieces once a week.

Sixty laboratory hatched tadpoles were bred at CAS (University of the Witwatersrand Central Animal Services) from a healthy male and a healthy female frog procured from the African Xenopus Facility. The female frog was injected with 250 iu gonadotropin (Sigma Aldrich, USA) hormone into the dorsal lymph sac to stimulate egg production and the male frog was injected with 50 iu gonadotropin 12 h after injecting the female. The eggs were fertilized by the male frog. The hatched tadpoles were acclimatized up to 10 days old (Nieuwkoop and Faber stage 48), randomly distributed into 8 groups of 15 and exposed in duplicate to 0 (control), 0.01, 200 and 500 $\mu g\,L^{-1}$ atrazine solutions (prepared in water) in 80 L glass tanks. Tadpoles were fed *ad libitum* with highly nutritious commercial ornamental fish micro flakes (TetraMini baby®) and received crushed nutritious commercial fish pellets (Koi food) as from 75 days old. The adult *X. laevis* and tadpoles were euthanized at 360 and 100 days old respectively.

2.2.1. Animal housing conditions

Activated carbon filtered and dechlorinated water was used to prepare the control and atrazine exposure water. Adult frogs and tadpoles were exposed in 60 L and 50 L, respectively of the 3 different atrazine concentrations (as well as control). The 30 $mg\,L^{-1}$ atrazine stock solution was added to the water in the 0.01, 200 and 500 $\mu g\,L^{-1}$ atrazine exposure tanks. The frogs and tadpoles were maintained at the CAS Lab with a 12 h light: 12 h dark cycle (on 06:00, off 18:00). Average water temperatures were maintained at 20 °C for adult frogs and average tadpole water temperatures were maintained at 22 °C utilising water heaters. Individual tank thermometers were verified using calibrated thermometers. The adult *X. laevis* were weighed every week and inspected for signs of stress or ill-health. Tadpole water was kept aerated by pumping air bubbles into the water through clean sand and wool filter using a pump. Quality control analysis of water in the 0.01, 200 and 500 $\mu g\,L^{-1}$ atrazine exposure tanks showed negligible

absorption of atrazine by the sand and wool. Adult frog atrazine exposure solutions were replenished 3 times a week after feeding to maintain a clean environment and to maintain atrazine levels. Water for tadpoles underwent a weekly 40% change for the first 4 weeks to reduce stress to the tadpoles and 100% afterwards. The frogs and tadpoles were euthanized by immersion in 0.2% benzocaine solution (an anesthetic) in Milli-Q water. This work was carried out under permit numbers CPF6 0115 (2015) and CPF6 0120 (2016) from Gauteng Nature Conservation. All experiments were performed in accordance with the regulations of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by the Animal Ethics Screening Committee with ethical clearance number 2014/32/D together with modifications and extensions granted.

2.2.2. Gravimetric measurements

The length of each tadpole was measured using a ruler placed under a transparent glass beaker containing the tadpole. The tadpole length was measured initially from snout-tail at 41 d old and from snout-vent thereafter, up to 100 d old. The tadpole mass at 41 d old was measured by water volume displacement as the tadpoles were stressed when taken out of the water. From 54 d old, the tadpole mass was measured accurately using a sensitive 4 digit balance as with adult frogs. Testicular volume (both testes) of the adult frogs was measured by water volume displacement and testicular mass (both testes) was measured using a sensitive 4-digit balance.

2.3. Histology

2.3.1. Differential staining for light microscopy

Testes were dissected immediately after euthanising, cut into 3 blocks and fixed in 2.5% gluteraldehyde. Blocks were selected randomly, processed and embedded in paraffin. 7 µm thick microtome sections were sectioned and mounted on slides. After deparaffinising and dehydrating, one set of sections was stained with hematoxylin and eosin (H & E). Another set was stained with Van Gieson stain for collagen.

2.3.2. Electron microscopy

Qualitative histological transmission electron microscopy (TEM) of testes from the first set of duplicate exposed adult *X. laevis* was conducted at the University of the Witwatersrand Microscopy and Microanalysis Unit. Quantitative TEM imaging work on testes of the second duplicate set of *X. laevis* was done at the Vrije University, Amsterdam (VU/VUmc) Electron Microscopy Facility. After euthanising the frogs, testes were immediately dissected, sectioned into 3 blocks and fixed in 2.5% gluteraldehyde (in 0.1 M calcium cacodylate ($C_4H_{12}As_2CaO_4$), pH 7) and left at room temperature for 1 h before refrigerating overnight at 4 °C. The testes blocks were washed twice in 0.1 M $C_4H_{12}As_2CaO_4$ buffer before transferring to shipping tube containing 30% sucrose in 0.1 M $C_4H_{12}As_2CaO_4$. The tubes were couriered to The Netherlands for processing and imaging.

Tissue blocks were trimmed to 1 × 3 mm blocks and postfixed overnight at 4 °C in fresh 2.5% gluteraldehyde (in 0.1 M $C_4H_{12}As_2CaO_4$). The tissue blocks were washed 3 times in 0.1 M $C_4H_{12}As_2CaO_4$ and postfixed with 1% OsO_4/1% $KRu(III)(CN)_6$. Blocks were washed in aqua bidest before dehydration by a series of increasing ethanol concentration (30%, 50%, 70%, 90%, 100%, followed by a second 100%, allowing the tissue block to sink to the bottom before moving to the next higher ethanol concentration). The tissue was washed with propylene oxide before infiltrating with propylene oxide: EPON (epoxy resin) (1:1, v/v) followed by propylene oxide: EPON (2:1 v/v) then embedded in freshly made EPON inside BEEM capsules filled with EPON. The blocks were allowed to sink down to the tip over 2 h. Polymerisation of the EPON was done at 65 °C for 48 h. The blockface was manually trimmed and 70 nm ultrathin sections were collected on formvar coated copper grids (without carbon) by room temperature ultramicrotomy using a diamond knife. The sections were double contrasted using Reynolds lead citrate and uranyl acetate, before analysing in a JEOL1010 TEM.

2.4. Chemical analysis

2.4.1. Serum extraction and cleanup

Immediately after euthanising, the adult frogs were bled out by making an incision on the subclavian artery and blood was collected using a syringe. Blood from 15 frogs was pooled together, allowed to clot for 15 min at room temperature, centrifuged to separate the serum and immediately stored at −82 °C prior to analysis. The serum samples were analysed for atrazine metabolites and steroid hormones. 100 µL of serum sample and 0.5 mL of saturated ammonium sulphate solution were added to an Eppendorf tube, together with 50 µL of 0.1 mg L^{-1} D7 deethylatrazine, 50 µL of 0.4 mg L^{-1} D4 17-β estradiol and 50 µL of 0.4 mg L^{-1} D4 estrone internal standards. The mixture was vortexed for 30 s before centrifuging for 8 min at 3800 g (4000 rpm). The clear top layer was removed and transferred to a test tube containing 200 mg PSA/C18 dispersive solid phase extraction (dSPE) sorbents for sample cleanup. 6 mL acetonitrile was added before the mixture was vortexed for 30 s and centrifuged at 3800*g* for 8 min. The organic extract was transferred to a clean test tube and evaporated to near dryness. Extracts for testosterone analysis were reconstituted in 200 µL of 10% methanol (Milli-Q water: Methanol, 90:10 v/v). Extracts for 17-β estradiol and 17-α estradiol were reconstituted by adding 100 µL of 100 nM $NaHCO_3$ Milli-Q water (pH 10.5) and 100 µL of 1 g L^{-1} dansyl chloride (in acetone: 100 nM $NaHCO_3$ Milli-Q water (pH 10.5), (1:1 v/v)) in an insert inside an LC vial. Due to the low ionization efficiency of 17-β estradiol and 17-α estradiol, with electro-spray ionization (ESI) in Liquid Chromatography-Mass Spectrometry (LC–MS/MS), a chemical derivatisation method was utilized to enhance the sensitivity from a limit of detection (LOD) from 15 ng mL^{-1} to 1 ng mL^{-1}. The derivatisation and LC–MS/MS conditions are described elsewhere (Rimayi et al., 2018a).

2.4.2. Adult frog liver extraction and cleanup

Adult frog liver samples were analysed for atrazine metabolites. 1 g (fresh weight (fw)) finely ground frog liver samples were weighed into 50 mL fluoroethylenepropylene centrifuge tubes. 5 g beef liver (used to feed adult frogs) purchased form a local supermarket was analysed along with the frog liver samples as a quality control (QC) sample. 50 µL of 0.1 mg L^{-1} D7 deethylatrazine internal standard was added to each sample. 4 mL acetonitrile (Sigma-Aldrich, Missouri, USA) and 0.2 g NaCl (Merck, Darmstadt, Germany) were added before manually shaking vigorously for 15 s, vortexing for 15 s at 35 Hz and allowing the mixture to equilibrate for 12 h. The samples were centrifuged for 15 min at 3800*g* and the upper organic layer was removed before adding another 4 mL acetonitrile, vortexing for 15 s at 35 Hz and centrifuging for 15 min at 3800 g. The second organic layer was removed and combined with the first organic layer in a clean centrifuge tube.

To each centrifuge tube containing a frog liver extract, a QuEChERS kit (containing 50 mg PSA, 50 mg C_{18}, 150 mg $MgSO_4$ (Agela Technologies, Delaware, USA)) was added together with an additional 200 mg $MgSO_4$ (Sigma-Aldrich, Missouri, USA), 200 mg PestiCarb (Agela Technologies, Delaware, USA), 100 mg diatomaceous earth (Sigma-Aldrich, Missouri, USA) and 250 mg basic alumina (Sigma-Aldrich, Missouri, USA). The centrifuge tubes were vortexed for 15 s at 35 Hz, left to settle for 5 min and then centrifuged for 8 min at 3800*g*. Each supernatant was transferred to a clean test tube and evaporated to near dryness using a gentle stream of nitrogen. All extracts were reconstituted in 200 µL of 10% methanol for LC–MS/MS analysis.

2.4.3. LC–MS/MS analysis

Analysis of testosterone, 17-β estradiol and 17-α estradiol and atrazine metabolites in serum and liver extracts was performed on an LC-ESI–MS/MS (1200 series LC system, 6410 triple quadruple MS;

Agilent Technologies, Amstelveen, The Netherlands). Analytical instrument conditions for steroid hormone and atrazine metabolite analysis are described by Rimayi et al. (2018a,b), respectively.

2.4.4. Gas Chromatography–Mass Spectrometry (GC–MS) analysis

Analysis of atrazine exposure water was performed by gas chromatography-mass spectrometry (GC–MS, GC 6890, MS 5975, Agilent Technologies, CA, USA). An Agilent Technologies HP-5MS (5% polydimethylsiloxane) column (30 m × 0.25 mm × 0.25 μm; Chemetrix, Johannesburg, South Africa) was used with an average velocity of 50 cm s^{-1}. GC oven temperature programming was 90 °C (2 min), 25 °C min^{-1}–200 °C (2 min), 8 °C min^{-1}–280 °C (3 min) with a runtime of 19.4 min. A splitless 1 μL injection was used at 240 °C. The limit of detection (LOD) of 0.002 μg L^{-1} and limit of quantification (LOQ) of 0.007 μg L^{-1} were estimated at 3x and 10x the signal to noise ratio respectively.

2.4.5. Quality control

The atrazine concentrations in the exposure tanks were measured weekly by GC–MS. 50 mL water samples were sampled before and after water change/recycle. The 50 mL water samples were extracted by passing through a 200 mg Bond Elut Plexa (Chemetrix, Johannesburg) solid phase extraction cartridge. All atrazine exposure water concentrations and were verified to be within ± 6% for 0.01 μg L^{-1}, ± 3.1% for 200 μg L^{-1} and ± 1.6% for 500 μg L^{-1} target concentrations. Atrazine was not detected in the control water. All tadpoles were fed the same quantity and quality of food and adult frogs were also fed the same quantity and quality of food. All tadpoles were kept at the same optimum temperatures and the adult frogs were kept at the same optimum temperatures as well. All chemicals used in this study were certified with a purity ≥97.5% with the exception of D7 deethylatrazine which had a purity of 96%. Analytical standards were supplied by Dr Ehrenstorfer and Toronto Research Chemicals (Industrial Analytical, Johannesburg).

2.4.6. Statistical analysis

Statistical analysis was performed using SPSS ver 16. One-way ANOVA analysis was applied for tadpole mass, length and mortality. Tukey Honestly Significant Difference (HSD) post-hoc analysis was used to determine which atrazine exposure concentrations (from this point, reference to atrazine exposure concentrations includes the control in all instances) differed significantly from others. A p-value ≤0.05 was considered statistically significant for all datasets. The Shapiro-Wilk test was applied to test for normality of data and the Levene's test for equality of variances was performed to determine if the differences in the exposure groups were due to random errors or differences between the variances of the frogs/tadpoles. For measurements taken only once, after sacrifice e.g testicular morphology, a one sample t-test was performed with the measurements sampled randomly and meeting the criteria for normal distribution. Data from the second adult frog exposure as well as the duplicate tadpole exposures was used for statistical analysis. Probit analysis software (PriProbit ver. 1.63) was used to compute LC50 values (the lethal dose at which 50% of a population dies within the 90 d exposure period).

3. Results and discussion

3.1. Frog and tadpole morphometry

3.1.1. Adult X. laevis and tadpole growth and motility

The adult *X. laevis* showed good health and weight gain within +3.1 g for the second atrazine exposure (Supplementary Information (SI) Table 1, n = 60) which was used for morphometric and histological analysis. Tadpoles in the control and 0.01 μg L^{-1} showed good health throughout the study with the 0.01 μg L^{-1} exposed tadpoles showing the highest growth rates in terms of weighed mass throughout the entire exposure period (SI Fig. S1). Only the tadpole mass and the adult frog testicular mass and volume met the assumption of normality (Table 1, Shapiro-Wilk test p-value > 0.05). All the mean adult frog masses from the different atrazine exposures were statistically different (Table 1, Tukey HSD p-value < 0.05). This result is influenced by the fact that the individual frogs within each atrazine exposure group had a different mass.

The mean tadpole masses of the different atrazine exposure concentrations were significantly different (p-value < 0.05 from one-way ANOVA) as the mean mass of the 500 μg L^{-1} exposed tadpoles was significantly lower than the mean masses of the control and 0.01 μg L^{-1} exposed tadpoles groups (Table 1, Tukey HSD p-value < 0.05). Differences in the tadpole lengths across all atrazine exposure concentrations were statistically insignificant (Table 1, p-value > 0.05 from one-way ANOVA; Tukey HSD p-values > 0.05 for all exposure concentrations). On observation, the 500 μg L^{-1} exposed tadpoles looked significantly smaller and thinner than the control, 0.01 μg L^{-1} and 200 μg L^{-1}exposed tadpoles throughout the study. Larvae in the 200 and 500 μg L^{-1} atrazine exposures showed signs of stress within 18 h of exposure, with reduced motility. The 0.01 μg L^{-1} exposed tadpoles had the highest average mass of 1.5 g as well as the longest average length of 3 cm throughout the exposure period with the 500 μg L^{-1} exposed tadpoles recording the lowest average mass of 0.8 g and average length of 2.5 cm (SI Figs. S1 and S2). The high atrazine concentrations therefore had a significant adverse effect on tadpole growth.

3.2. Frog and tadpole mortality rates

Mortality rates for adult frogs were 0% for all exposure concentrations. Mortality rates of the 500 μg L^{-1} exposed tadpoles were significantly higher than all the other exposure concentrations (Table 1, p-value < 0.05 from one-way ANOVA). Mortality rates for tadpoles were 0, 0, 3.3 and 70% for the control, 0.01, 200 and 500 μg L^{-1} respectively exposed, tadpoles for the 90 day exposure period (Fig. 1, n = 120) and did not follow a normal distribution (Table 1, Shapiro-Wilk test p-value < 0.05). Four tadpoles in the 500 μg L^{-1} exposure tanks died within 18 h of exposure and more gradually continued to die during the 90 day exposure period in both duplicate tanks. Only one death was recorded in one of the duplicate 200 μg L^{-1} atrazine exposure tanks. Similar dose dependant mortalities were discovered in a study by Ji et al. (2016). As with tadpoles exposed to 400 μg L^{-1} atrazine concentrations by Langerveld et al. (2009), the tadpoles exposed to 500 μg L^{-1} experienced high mortality rates.

A 90 d LC50 of 343.7 μg L^{-1} was computed using Probit analysis software for tadpoles exposed to the different atrazine concentrations (0–500 μg L^{-1} exposure range) from 10 days old (Stage 48 Niewkoop and Faber stage). A general LC50 of 410 μg L^{-1} has been suggested for amphibians (Solomon et al., 2008). Atrazine concentrations ≥343.7 μg L^{-1} in breeding sites can therefore be considered catastrophic to pre-metamorphosis tadpoles as they may face significant survival risks at this concentration.

3.3. Metamorphosis

The high atrazine concentrations utilized did not significantly influence metamorphosis rates of tadpoles (p-value > 0.05 from one-way ANOVA). The metamorphosis for the control, 0.01 μg L^{-1} and 500 μg L^{-1} exposed tadpoles was complete within 44–50 days, whilst the 200 μg L^{-1} exposed tadpole metamorphosis took longer to complete at 58 days (Fig. 2). The 500 μg L^{-1} atrazine exposed tadpoles experienced very high mortalities which introduced bias in the metamorphosis rate, as the surviving 20 out of 30 tadpoles completed metamorphosis within 44 days. The data shows a general trend that atrazine increases the time to metamorphosis, though statistically insignificant. It has been previously reported by Solomon et al. (2008) that atrazine exposures of 20–200 μg L^{-1} had no effect on development rate, percent

Table 1
Frog and tadpole morphometry statistical analysis.

Parameter	N	Shapiro-Wilk *p*-value	Levene's *p*-value	One-way ANOVA *p*-value	Post-hoc analysis Tukey HSD *p*-value	T-test *p*-value
Adult frog mass	60	0	0.364**	0***	***$p < 0.05$ all cases	–
Adult frog testicular mass	60	0.51*	–	–	–	1
Adult frog testicular volume	60	0.555*	–	–	–	0.816
Tadpole mass	120	5.35*	0.65**	0.002***	***$p < 0.05$ for 500 µg L^{-1} exposed tadpoles	–
Tadpole length	120	0	0.994**	0.97	$p > 0.05$ all cases	–
Tadpole mortality	120	0	0.045	0.01***	ϕ	–

ϕTukey HSD invalid because control and 0.01 exposures both have a variance of 0.
– Test not applicable.
All cases = all atrazine exposure concentrations.
* Normal distribution assumed.
** Equal variances assumed.
*** Significant at α = 0.05 probability.

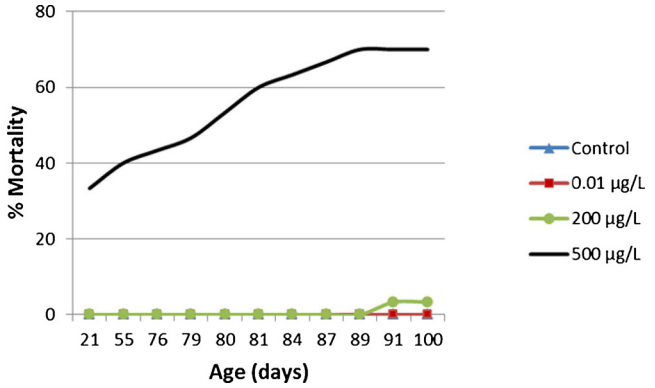

Fig. 1. Tadpole mortality rate timeline after exposure to different atrazine concentrations (n = 120).

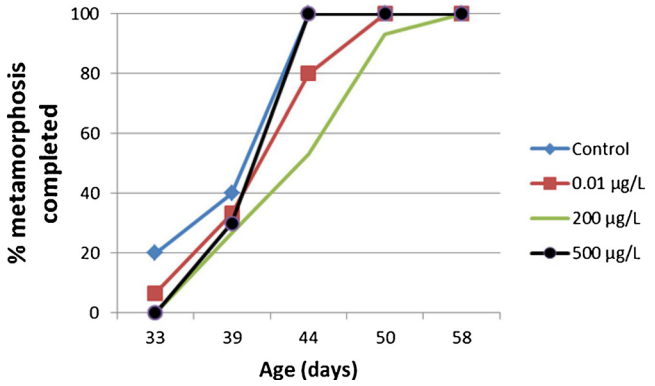

Fig. 2. *X. laevis* tadpole metamorphosis after exposure to different atrazine concentrations (n = 120).

metamorphosis and time to metamorphosis. Another study by Freeman and Rayburn, (2005) and references therein indicate that atrazine increases the time to complete metamorphosis.

3.4. Testicular morphology, mass and volume of adult frogs

There were no statistically significant differences between the adult frog testicular masses (Table 1, one sample *t*-test, T = 0, df = 3, mean = 0.116, *p*-value > 0.05) and volumes (one sample *t*-test, T = −2.54, df = 3, mean = 0.1683, *p*-value > 0.05) recorded between the different atrazine exposure concentrations. The average testicular mass and volume of the control (0.193 g and 0.178 cm^3 respectively) and 0.01 µg L^{-1} exposed frogs (0.167 g and 0.123 cm^3 respectively) were however higher than the 200 µg L^{-1} exposed frogs (0.083 g and 0.11 cm^3 respectively) and 500 µg L^{-1} exposed frogs (0.095 g and

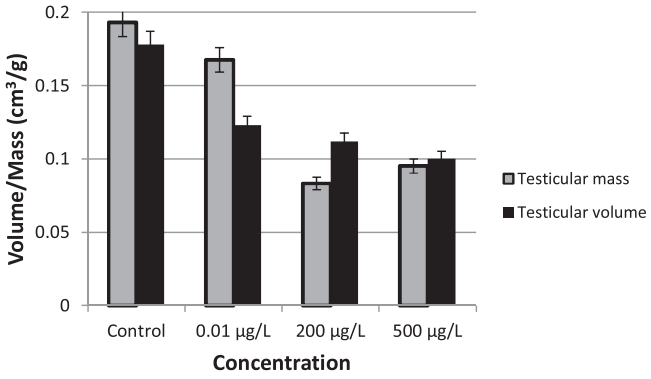

Fig. 3. Adult *X. laevis* average testicular volume and mass (error bars = standard deviation) measured after atrazine exposure at different atrazine concentrations (n = 60).

0.1 cm^3, respectively) (Fig. 3). The average testicular mass of the 500 µg L^{-1} exposed frogs was slightly higher than that of the 200 µg L^{-1} exposed frogs, exhibiting a non-monotonic response. The deleterious effect of atrazine on developing *X. laevis* tadpoles exposed for just 48 h has been described in great detail by Tavera-Mendoza et al. (2002), with follow-up work carried out by Oka et al. (2008) and therefore do not warrant further investigation for much longer exposures such as 90 days.

The natural phenomenon or otherwise, resulting in occurrence of multiple testes and hermaphrodites as described by Coady et al. (2004); Hayes et al. (2002); Hayes, (2005); Carr et al. (2003); Jooste et al. (2005) and Oka et al. (2008) was not detected in any of the 120 adult male frogs from the two frog batches utilised in this exposure study. This may in part be due to the fact that adult frogs in this study had already developed healthy normal testes in a pristine environment before the atrazine exposure.

3.5. Histology

Qualitative histological assessment of duplicate sets of atrazine exposed adult frogs (exposed at different times) showed similar testis histological morphology in the respective atrazine exposure concentrations. Histological morphology of control adult frogs from both groups showed similar histology but showed a range of differences from the atrazine exposed testes.

3.5.1. Adult X. laevis germ cell line

The germ cell line in control frogs was well ordered and well-structured as differentiated cells could be distinguished from others in the sequential order spermatogonium- primary spermatocyte- secondary spermatocyte- spermatid (Fig. 4A). The 0.01 µg L^{-1} exposed frogs also showed normal germ cell development. The 200 µg L^{-1} and

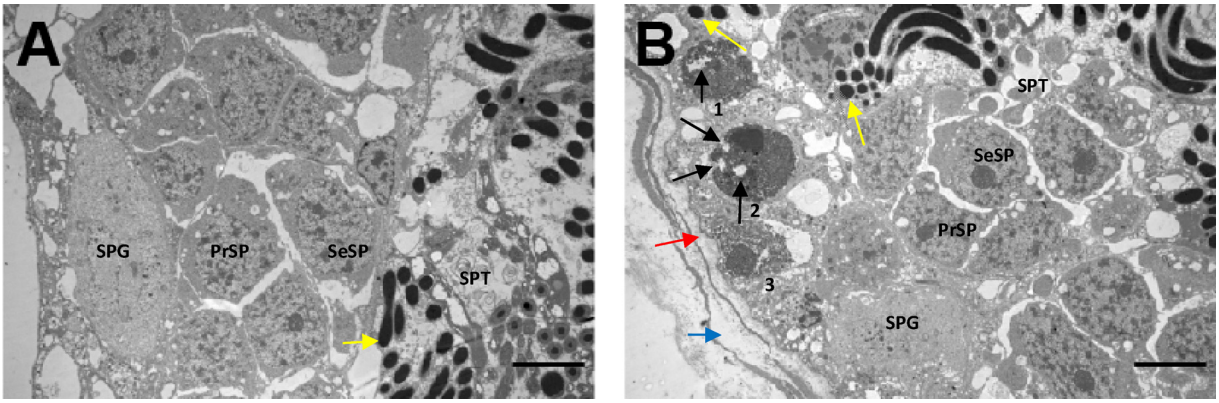

Fig. 4. Electron micrograph showing germ cells in *X. laevis,* A- control, B- 500 μg L^{-1} atrazine exposure. Scale bar = 5 μm. SPG = spermatogonium; PrSP = primary spermatocyte; SeSP = secondary spermatocyte; SPT = spermatid; yellow arrow = spermatozoa; black arrow = vacuole in Sertoli cell nucleus, red arrow = basal lamina; blue arrow = smooth muscle (basement membrane). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

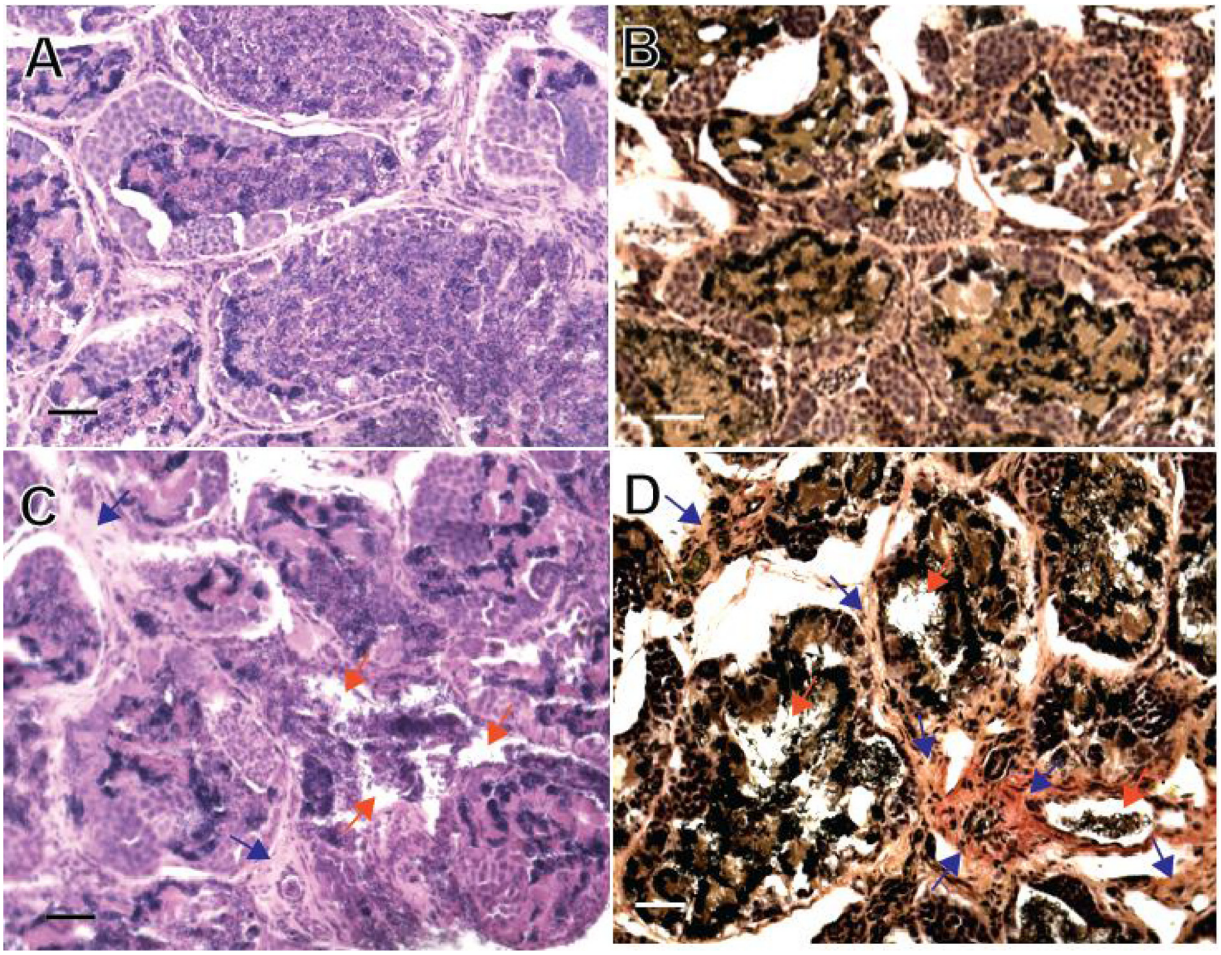

Fig. 5. Typical seminiferous tubules with H&E staining, control (A) and 500 μg L^{-1} atrazine exposures (C), Van Gieson staining control (B) and 500 μg L^{-1} atrazine exposures (D). Scale bar = 70 μm, 20 x magnification. Red arrows show empty spaces in seminiferous tubules and blue arrows show abnormal amounts of connective tissue which are more visible with Van Gieson staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

500 μg L^{-1} atrazine exposed frogs showed a high incidence of seminiferous tubule degradation with total or occasional significant disruption of the germ cells lines (Fig. 4B). The 500 μg L^{-1} atrazine exposed seminiferous tubules showed significant abnormalities such as Sertoli cells (Fig. 4B cells 1, 2 & 3) clustered towards the basal lamina (red arrow) not extending towards the lumen of the seminiferous tubule and with no spermatogonia between them. As a result, spermatids (yellow arrows) were located in many different sections of the seminiferous tubule such as near the basal lamina (Fig. 4B, red arrow) instead of in the central lumen of the seminiferous tubule. The seminiferous tubule histological abnormalities and deformities are consistent with findings by Hayes et al. (2011) but contrary to findings

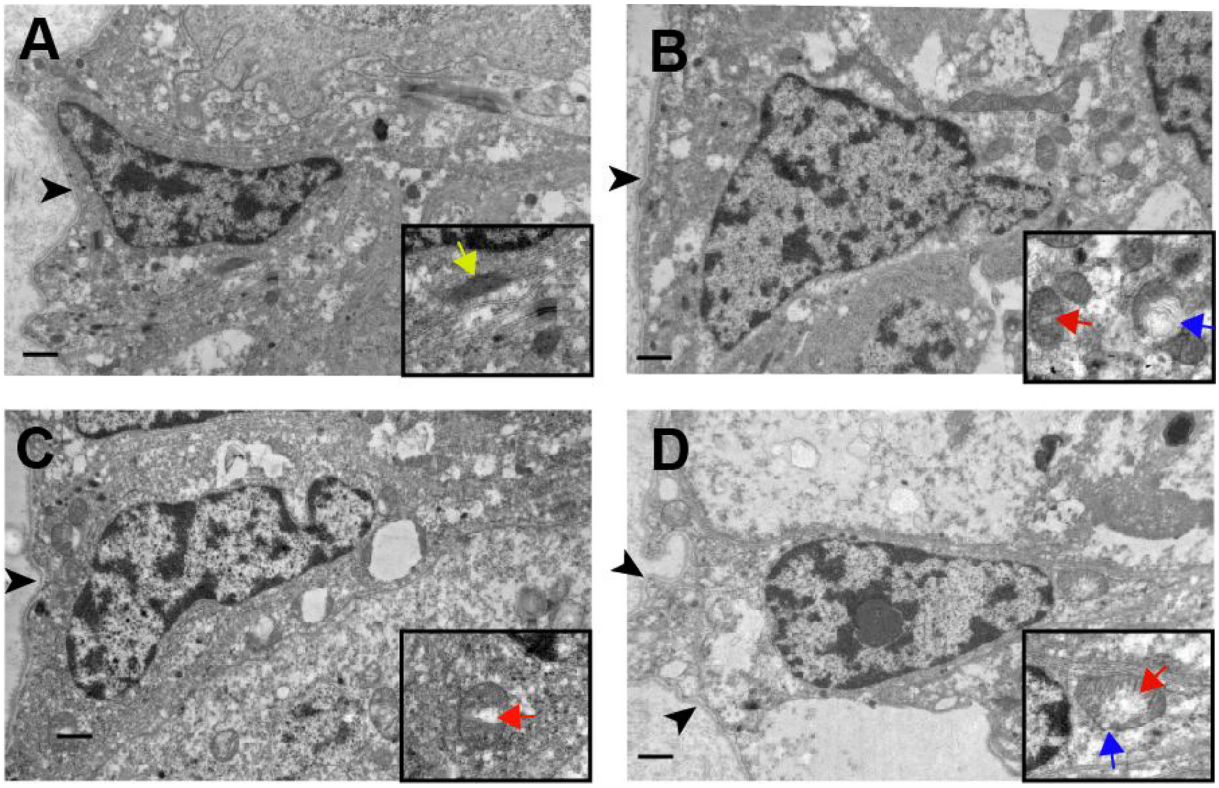

Fig. 6. Electron micrograph showing typical Sertoli cells of adult *X. laevis.* A- control, B- 0.01 μg L^{-1}, C–200 μg L^{-1}, and D- 500 μg L^{-1} atrazine exposure. Scale bar = 1 μm. Black arrowheads = the basal membrane, blue arrows = ruptured mitochondrial double membrane; yellow arrow = elongated healthy mitochondria; red arrows = vacuole in mitochondrial matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by Hecker et al. (2006) who found no evidence of ultrastructural destruction in germ cells. Nucleus-vacuole junctions (black arrows in Fig. 4B, sertoli cells 1 & 2) could be observed in the 500 μg L^{-1} atrazine exposed frogs, a sign of cell stress which could result in gonadal dysfunction.

The absence of testicular ovarian follicules (TOFs) in any of the 120 frogs tested in this study concurs with conclusions in the meta-study by Rohr and McCoy, (2010) that development of TOFs in adult frogs is not a natural occurrence. This alludes that the TOFs detected by various researchers cited by Hecker et al. (2006) in both test and control adult *X. laevis* may certainly have been due to other external factors inevitably introduced, particularly during the embryo and larval stages (Hayes et al., 2003). Atrazine contamination is particularly widespread in some laboratory experiments, microcosms, mesocosms and field studies as described by Hayes, (2005).

3.5.2. Adult X. laevis seminiferous tubule histology

Testes of frogs exposed to 200 and 500 μg L^{-1} atrazine concentrations had a significant degree of gonadal atrophy with poorly developed seminiferous tubules (Fig. 5C and D). Seminiferous tubule damage in 200 and 500 μg L^{-1} atrazine exposed frogs is consistent with findings by Chen et al. (2015) and Hayes et al. (2011) as the seminiferous tubules showed signs of disintegration with presence of hollow spaces within seminiferous tubules (Fig. 5C and D, red arrows). Van Gieson staining revealed significant fibrosis between seminiferous tubules of the 200 and 500 μg L^{-1} atrazine exposed frogs (Fig. 5D, blue arrows). This may cause the reproductive dysfunction and failure to reproduce. The effects of atrazine on adult *X. laevis* seminiferous tubule histology are similar to those reported by Hayes et al. (2010) for *X. laevis* exposed throughout the larval stage.

There are no statistically significant differences between the seminiferous tubule diameters of all atrazine exposure concentrations (Table 1, one sample *t*-test, *p*-value > 0.05), however control frogs recorded the longest average seminiferous tubule diameter of 356 μm. The 0.01 μg L^{-1} atrazine exposed frogs recorded a longer average seminiferous tubule diameter of 302 μm than the 200 μg L^{-1} and 500 μg L^{-1} atrazine exposed frogs of 231 and 237 μm respectively (SI Fig. S3).

3.5.3. Adult X. laevis sertoli cell ultrastructure

The health of the Sertoli cells was assessed by counting the number of mitochondria (n = 82-86 images) and damaged mitochondria present (n = 79-86 images). Stressed mitochondria often produce mitochondrial-derived vesicles (MDV) in response to stress inducing conditions (Soubannier et al., 2012; Sugiura et al., 2014). Sertoli cells of frogs exposed to atrazine showed high incidences of mitochondrial stress and damage as the 0.01, 200 and 500 μg L^{-1} exposed frog Sertoli cell mitochondria appeared vesicular (Fig. 6B–D respectively, red arrows show vesicles) with incidences of double wall rapture (Fig. 6 blue arrows), compared to control mitochondria which appeared elongated (Fig. 6A, yellow arrow).

The Sertoli cells of the 500 μg L^{-1} exposed frogs had significantly lower number of mitochondria, averaging approximately only 3.5 per field of view compared with the control, 0.01 and 200 μg L^{-1} which recorded an average of > 5 mitochondria per field of view (Fig. 7A). The Sertoli cells of the 500 μg L^{-1} exposed frogs had a very high average percentage of damaged mitochondria of 61% compared to the control, 0.01 and 200 μg L^{-1} recording lower values of 9, 25 and 45% respectively (Fig. 7B). As sertoli cell mitochondria have a central role in germ cell development, this function may be impaired due to high incidences of mitochondrial stress and damage.

3.5.4. Adult X. laevis leydig cell ultrastructure

Leydig cells could be identified by their irregularly shaped

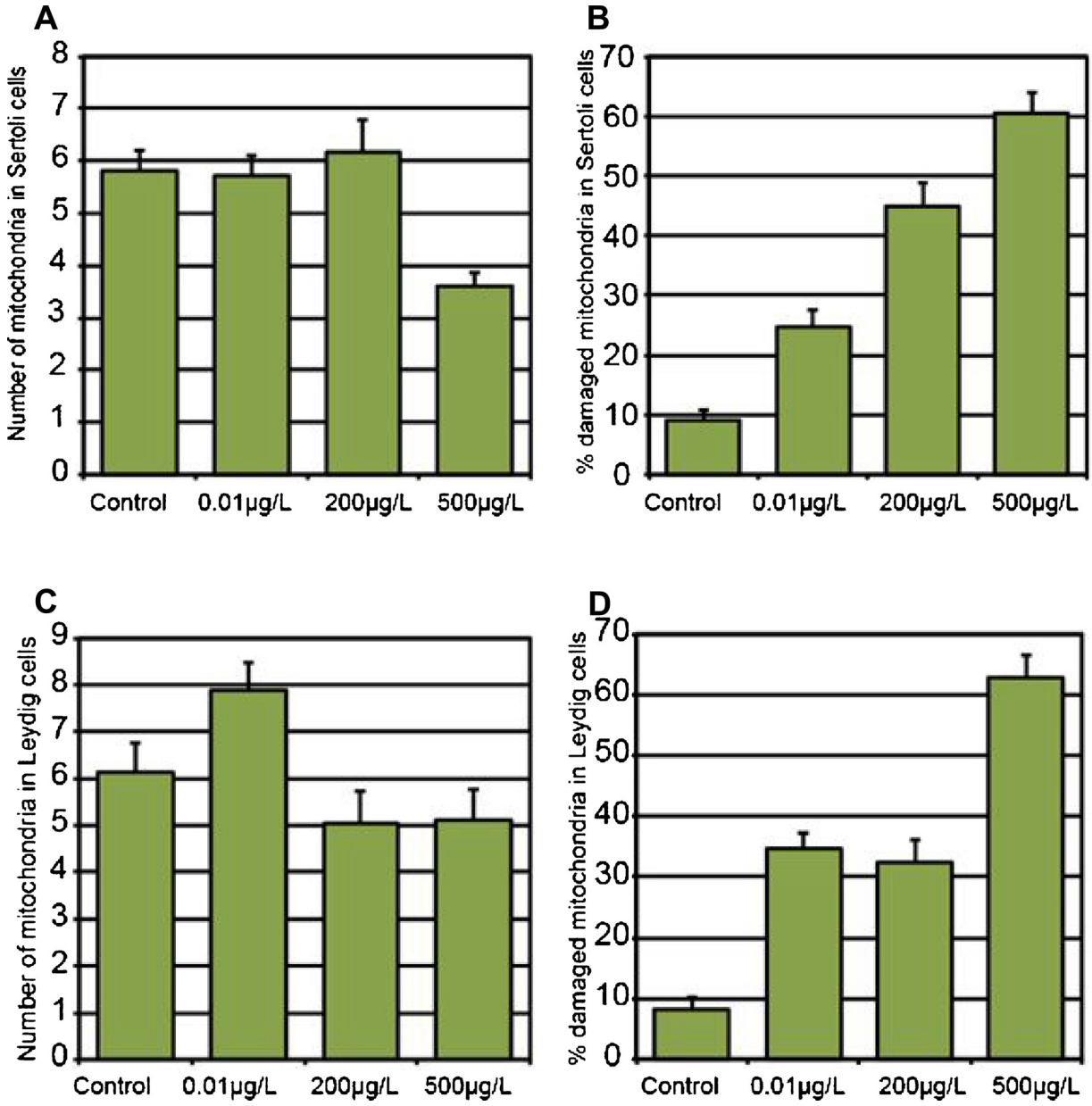

Fig. 7. Analysis of mitochondria in adult *X. laevis* Sertoli cells for each exposure concentration (A- average number of mitochondria and B- average number of damaged mitochondria). Analysis of mitochondria in adult *X. laevis* Leydig cells for each exposure concentration (C- average number of mitochondria and D- average number of damaged mitochondria) after atrazine exposure at different concentrations (error bars = standard deviation).

cytoplasm and as cells that lie outside the periphery of the seminiferous tubule. The Leydig cells are important in male sexual development as they are the primary testosterone producing cells (Hecker et al., 2005), with synthesis of testosterone in the Leydig cell being mediated in mitochondria and smooth endoplasmic reticulum (Kim et al., 2016). The health of the Leydig cells was assessed by quantifying the number of mitochondria (n = 61-79 images) as well as the percentage of damaged mitochondria (n = 59-76 images, Fig. 7C and D). The Leydig cells of the 200 and 500 $\mu g\,L^{-1}$ atrazine exposed frogs showed a significantly lower average number of mitochondria than the control and 0.01 $\mu g\,L^{-1}$ exposed frogs (Fig. 7C). The 500 $\mu g\,L^{-1}$ exposed frogs had significantly higher percentages of damaged mitochondria, averaging 63% compared to 35 and 32% for the 0.01 and 200 $\mu g\,L^{-1}$ exposed frogs (Fig. 7D). Damaged mitochondria in Leydig cells were characterized by swollen and vesicular appearance (Fig. 8, red arrows). Cytosolic lipid droplets in the Leydig cells of the 200 and 500 $\mu g\,L^{-1}$ atrazine exposed frogs (Fig. 8C and D respectively, black arrows) were significantly smaller than cytosolic lipid droplets of the control and 0.01 $\mu g\,L^{-1}$ exposed frogs. This can be attributed to suppression of steroidoegenesis in the synthesis of testosterone as healthy Leydig cell cytoplasms are typically rich in large lipid droplets (Fig. 8A and B [control and 0.01 $\mu g\,L^{-1}$ exposed frogs respectively]).

3.5.5. Rough endoplasmic reticulum (RER)

The RER in Sertoli cells, Leydig cells and germ cells of all the control and atrazine exposed adult *X. laevis* appeared similar with no signs of swelling. The RER in smooth muscle of 500 $\mu g\,L^{-1}$ appeared swollen (SI, Fig. S4 B) when compared to other treatments, however the incidence of swelling was not consistent within the cells and between different frog testes. RER in both Leydig cells (SI, Fig. S4C and D) and Sertoli cells showed no noticeable differences between control and 500 $\mu g\,L^{-1}$ exposures.

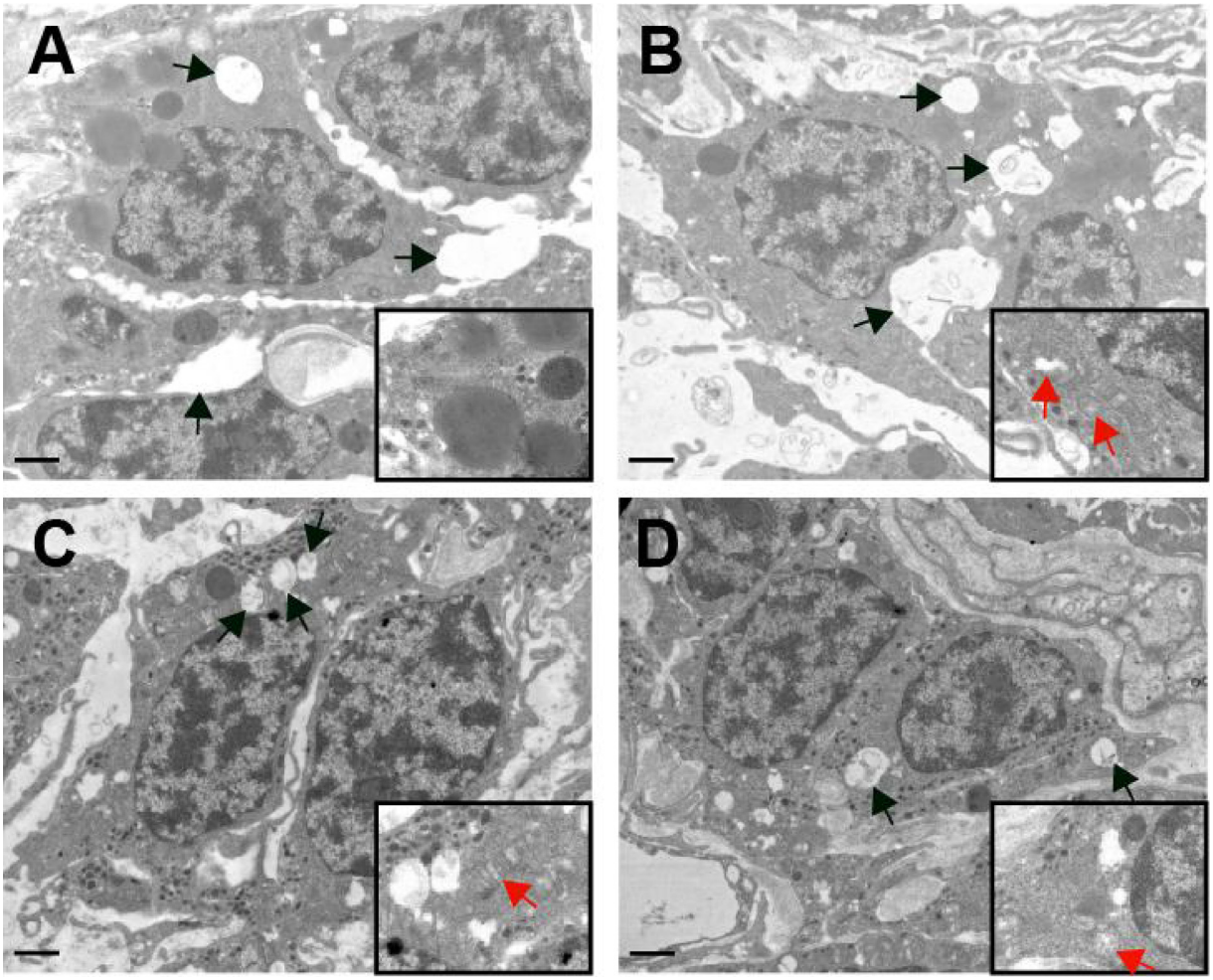

Fig. 8. Electron micrograph showing typical Leydig cells in adult *X. laevis*. A- control, B- 0.01 μg L^{-1}, C–200 μg L^{-1}, D- 500 μg L^{-1} atrazine exposure. Scale bar = 1 μm; Insert zoom shows mitochondria. Black arrows = lipid droplets, red arrows = vesicle/vacuole in mitochondrial matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Atrazine metabolites and testosterone levels (average ng mL^{-1} and standard deviation) measured in *X. laevis* serum (n = 15) after exposure to different atrazine concentrations.

Exposure concentration (μg L^{-1})	ADD (ng mL^{-1})	AD-2OH	DIA	A-2OH	DEA	Testosterone
500	16.5 ± 2.3	0.8 ± 0.2	72 ± 3.6	2.1 ± 1	78.5 ± 2.7	0.4 ± 0.1
200	9.9 ± 1.5	0.2 ± 0.1	28.8 ± 2	1.9 ± 0.4	27.8 ± 1.4	0.6 ± 0.3
0.01	N.D	N.D	N.D	3.8 ± 0.3	0.6 ± 0.1	1.1 ± 0.5
Control	N.D	N.D	N.D	N.D	N.D	1.3 ± 0.6

N.D = Not detected.

Table 3
Atrazine metabolites measured (average ng g^{-1} fresh weight and standard deviation) in frog liver (n = 5) after exposure to different atrazine concentrations, and unexposed QC sample (beef liver).

Exposure concentration (μg L^{-1})	ADD	AD-2OH	DIA	HA	DEA
	(ng g^{-1} fresh weight)				
500	1.9 ± 0.6	0.3 ± 0.1	26.5 ± 1.2	0.9 ± 0.2	59.7 ± 6.7
200	1.3 ± 0.4	0.4 + 0.2	15.6 ± 2.6	0.9 ± 0.4	42.1 ± 4.3
0.01	N.D	N.D	N.D	N.D	N.D
0.00	N.D	N.D	N.D	N.D	N.D
QC sample	N.D	N.D	N.D	N.D	N.D

N.D = Not detected.

3.6. Biochemical analysis

The serum testosterone concentration of the 0.01 μg L^{-1} atrazine exposed frogs was marginally lower than the control. The serum testosterone concentrations of the 200 μg L^{-1} and 500 μg L^{-1} atrazine exposed frogs were 2 and 3 times lower than control, respectively (Table 2). This suggests a graded dose effect as reported by Hayes et al. (2010). 17-β estradiol and 17-α estradiol in serum were below the limit of detection after derivatisation, hence could not be detected. The endocrine disrupting effects of atrazine described by Hayes et al. (2002) were observed in this study.

The presence of atrazine metabolites in frogs is not well documented and data is scanty (Solomon et al., 2008). *In vitro* tests have shown that DEA and DIA induce aromatase activity with the same potency as

atrazine (Sanderson et al., 2001). All 6 atrazine metabolites tested were detected in either serum or liver of frogs exposed to 200 and 500 μg L^{-1} atrazine concentrations. For frogs exposed to 500 μg L^{-1} atrazine concentrations, the relative concentrations of ADD, AD-2OH, DIA and DEA in serum (Table 2, 16.5 ± 2.3, 0.8 ± 0.2, 72 ± 3.6 and 78.5 ± 2.7 ng mL^{-1} respectively) were all higher than the relative concentrations in the liver (Table 3, 1.9 ± 0.6, 0.3 ± 0.1, 26.5 ± 1.2 and 59.7 ± 6.7 ng g^{-1}, respectively). The same trends existed for the 200 μg L^{-1} atrazine exposed frogs with the exception of AD-2OH and DEA which recorded higher concentrations in the liver. Atrazine metabolism in humans and animals has been described as a complex process which can vary from individual to individual (Joo et al., 2010). Only A-2OH and DEA were detected in serum from frogs exposed to 0.01 μg L^{-1} atrazine concentrations. Atrazine metabolites were not detected in both serum and liver of control frogs, liver of frogs exposed to 0.01 μg L^{-1} atrazine concentrations as well as beef liver quality control (QC) sample (Tables 2 and 3). DIA and DEA recorded the highest concentrations in both frog serum and liver (Tables 2 and 3), indicating that they are the major metabolites. High concentrations of 78.5 ng mL^{-1} for DEA in serum, as well as 59.7 ng g^{-1} in liver of the 500 μg L^{-1} atrazine exposed frogs may indicate that there is a significant degree of atrazine uptake and metabolism.

4. Conclusions

Exposure of *X. laevis* tadpoles to high sub-chronic atrazine concentrations of 500 μg L^{-1} lead to a significant reduction in tadpole mass and a significant increase in tadpole mortality, whilst exposure to 0.01 and 200 μg L^{-1} atrazine concentrations did not lead to significant mortality or reduction of tadpole growth and development. Exposure of adult *X. laevis* to 200 and 500 μg L^{-1}atrazine concentrations lead to seminiferous tubule structure damage and gonadal atrophy, with significant disruption of normal germ cell lines. Atrazine also caused significant stress and damage to Sertoli and Leydig cell mitochondria and as a result led to diminished serum testosterone levels in the atrazine treated groups. The data generated indicates that environmentally relevant atrazine concentrations adversely affect frog gonadal development and tadpole survival at concentrations above the calculated LC50 value of 343.7 μg L^{-1}.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.03.028.

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